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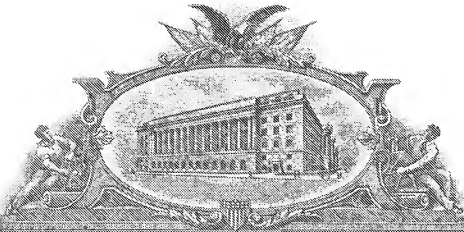
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Catalytic generation of biomarkers from bacterial endospores

Calvin. H. Bartholomew¹, K. Narasimharao¹, Phillip Smith¹, Milton Lee²

¹Department of Chemical Engineering

²Department of Chemistry
Brigham Young University
Provo, UT 84602

Biological Warfare Agent Detection: Introduction and Background

The increase in production and availability chemical and biological warfare agents throughout the world has resulted in a serious threat of attack on the United States of America. Of specific concern is agent such as *Bacillus anthracis* (anthrax). The advent of this threat highlights the need for methods to rapidly detect and identify such biological warfare agents [1]. There are several methods used for such identification, nucleic acid sequencing (deoxyribonucleic acid, DNA or ribonucleic acid, RNA), which uses polymerase chain reactions (PCR) or culture growth. This method is currently the method of choice due to its high specificity; however, it requires a significant amount of laborious work to accurately sequence the nucleic acids and may require required several days to identify the biological agent. [2].

A number of other methods have been developed which focus on the detection of calcium-complexed dipicolinic acid and its derivatives. Calcium-complexed dipicolinic acid (DPA) is a major constituent of *Bacillus* endospores (10-15% of dry weight). Relatively fast methods have been developed to chemically extract the calcium-complexed DPA and then detect it or its acid form, by mass spectrometry [3], luminescence [4] or fluorescence [5]. Although luminescence and fluorescence methods provide a relatively high degree of discrimination and sensitivity, they still require significant time due to sample handling and analysis requirements. Another method used for the detection of *Bacillus* endospores employs either a quartz frit (filter) to collect aerosol spores or a small wire onto which liquid dissolved spores are deposited and dried. Following collection or deposition, the spores are pyrolyzed at temperatures between 500 and 650°C. Gas chromatography is used to separate the volatilized components, and a time-of-flight ion mobility spectrometer is used to detect the biomarkers produced which include DPA and its pyrolysis derivatives [6, 7]. This pyrolysis method is fast (on the

order of minutes), but requires high temperatures with associated high energy requirements and low reaction selectivity. Thus, a significant problem is the production of numerous by-products, which increase the difficulty of interpreting the final spectra. Pattern recognition algorithms have been employed to aid in this interpretation, increasing the complexity of the system. The detection of calcium-complexed DPA and its derivatives is a valid approach to identify a class of spores but only for those bacterial endospores that contain DPA.

Each of the above mentioned methods have serious limitations in their ability to rapidly detect biological warfare agents. These limitations include longer detection times, large equipment/volume requirements, high temperatures, large energy needs, and complicated data analysis routines. While these methods are currently used in many large scale applications, the development of a fast, low energy requirement and portable detection system that free of the above limitations is needed.

Application of Catalyst Technology to Biological Warfare Detection

Catalysts have many different uses in industry and pollution abatement. Their major benefits are three-fold. First, because they facilitate reaction at low temperatures and pressures, they dramatically lower the energy requirements for chemical reactions and processes. Second, they offer tremendous increases in selectivity for a desired product or set of products. Third, they reduce the required equipment volume, and thus cost.

The application of catalyst technology to the rapid detection and identification of biological and chemical warfare agents has the capability to overcome the limitations of the current detection and identification methods discussed above. Specifically, the generation of specific biomarkers from DPA and other components of endospores using heterogeneous catalysts could be expected to (1) lower the consumption of energy, (2) selectively produce biomarkers, which would decrease the complexity of the identification process, and (3) reduce the size of required equipment and time for completion of the process. In this application, the catalyst breaks down and converts the biological material to detectable biomarkers. This biological material could include (but is not limited to) bacterial endospores, vegetative bacteria, viruses, toxins, and proteins.

A search of the scientific literature and patents indicates that currently there are no available reports on catalysts that can break down or convert biological materials to specific biomarkers. Therefore, this catalyst technology must be developed.

Development of Catalyst Technology:

To develop catalyst technology necessary to produce biomarkers from biological material, a literature study was performed. It was found that, homogenous esterification of DPA can be performed efficiently using soluble super acid catalysts at lower reaction temperatures [8]. But there are several disadvantages with a homogeneous reaction strategy such as dissolution of catalyst in the solvent and the need to isolate the product, the latter of which is typically laborious process and time consuming.

We propose the use of a heterogeneous solid super acid catalyst to esterify DPA. Conducting the reaction under heterogeneous conditions has several advantages including (1) time consumption in separation of products from catalysts and (2) gas phase reaction products are easily and quickly transported to and analyzed by GC/MS.

We demonstrate specifically here how solid super acid catalyst technology can be combined with an ethanol solvent as well as esterifying agent to produce DPA esters that are readily analyzed in the gas phase.

Experimental:

Dipicolinic acid (2,6-pyridinedicarboxylic acid, DPA), 3-pyridinecarboxylic acid, and 12-tungstophosphoric acid (TPA) were used as received from Sigma-Aldrich. Silica (catalyst grade) was purchased from Grace Davison, USA. Ethanol (AR grade) was used as an esterifying agent. Silica supported 12-tungstophosphoric acid catalysts were prepared by the conventional impregnation technique. A laboratory dual catalytic test reactor system was designed and constructed to evaluate performance of various catalysts. 0.5g of catalyst was placed between two pyrex glass wool plugs in the reactor. 10 mg of DPA was dissolved in 25ml of ethanol and pumped with a micro liter syringe pump at a fixed rate into a vaporizer, held at a temperature between 225 and 285°C. The generated vapor was carried by air (carrier gas, 5 sccm) into the reactor. The reaction

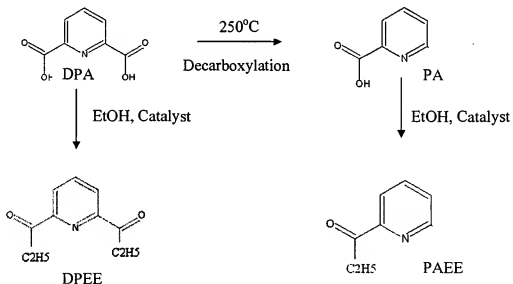
was carried out at temperatures of 250 and 300°C. The product distribution was analyzed by HP 5973 gas chromatograph coupled with mass detector.

Results & Discussion:

The reaction studied is the esterification of dipicolinic acid with ethanol. The influence of various reaction conditions are examined: the reaction is carried out under heterogeneous conditions using silica supported 12-tungstophosphoric acid (TPA) as a catalyst. One objective is to investigate reactions under different conditions at different TPA acid loadings from 10-50 wt% and effects of reaction temperature typically 250-300°C.

The reaction pathway is shown in the scheme 1. DPA is decarboxylated to 2-picolinic acid (PA) at 250°C and gas phase reaction between PA and ethanol gives the 2-picoloni acid ethyl ester (PAEE). Other step involved is direct transformation of DPA to dipicolinic ethyl ester (DPEE).

Table 1 shows that the activity of different catalysts for esterification of DPA with ethanol. With increase of loading the conversion is increasing and the selectivity to PAE is increasing. Increase of acidity of the catalysts with in TPA loading leading to more decarboxylation occurs and forming PAE.



Scheme 1: Esterification of dipicolinic acid with ethanol

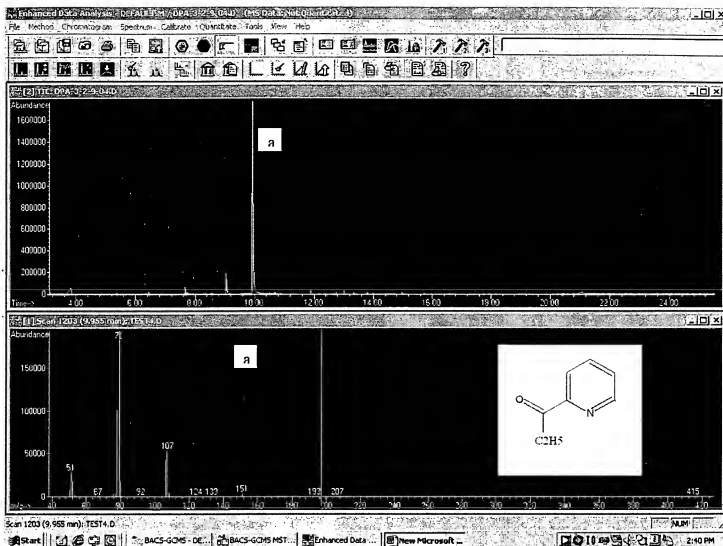


Fig 1: Typical GC/MS spectrum of product

Figure 1 shows the typical GC/MS spectrum of the product. The major peak (a) is corresponding to PAEE. We confirmed the product by matching the mass spectrum of this peak with mass spectrum in NIST library. These results are revealing that the catalysts can be used for esterification of DPA.

Table 1: Conversion and selectivity data of esterification of DPA with ethanol on different catalysts

Catalyst	Reaction temperature (°C)	Conversion of DPA	Selectivity to PAEE (%)	Selectivity to DPEE (%)
TPA	250	N/A	N/A	N/A
SiO ₂	250	N/A	N/A	N/A
10wt%TPA/SiO ₂	250	N/A	N/A	N/A
20wt%TPA/SiO ₂	250	N/A	N/A	N/A
30wt%TPA/SiO ₂	250	70	90	10
40wt%TPA/SiO ₂	250	79	95	5
50wt%TPA/SiO ₂	250	85	100	0

Conclusions:

Vapor phase esterification of DPA has been successfully carried out on supported solid acid catalysts. It is found that the catalysts successfully esterify DPA with high selectivity to PAE. These results indicating that DPA present in endospores can be converted to its corresponding esters (biomarkers) by using these catalysts. These results demonstrate in general how a catalyst can be used for rapid identification of endospores under mild conditions of low energy requirements, facilitating use in a highly portable system. It is specifically demonstrated how solid super acid catalyst technology can be combined with an ethanol solvent as well as esterifying agent to produce DPA esters that are readily analyzed in the gas phase.

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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53 (b)(2).

DOCKET NO.	04-04	TYPE PLUS SIGN	+
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INVENTOR(s)/APPLICANT(s)

LAST NAME	FIRST NAME	MIDDLE INITIAL	RESIDENCE (CITY, STATE)
Bartholomew	Calvin	H	Orem
Narasimharao	Katabathini		Provo
Smith	Phillip	L	Provo
Lee	Milton		Pleasant Grove

TITLE OF THE INVENTION (280 CHARACTERS MAX)

Catalytic Generation of Biomarkers from Bacterial Endospores

CORRESPONDENCE ADDRESS

Technology Transfer Office
Brigham Young University
A-285 ASB P.O. Box 21231
Provo

STATE	Utah	ZIP Code	84602-1231	COUNTRY	U.S.A.
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ENCLOSED APPLICATION PARTS (check all that apply)

<input checked="" type="checkbox"/> Specification	Number of Pages	7	<input checked="" type="checkbox"/> Applicant claims small entity status (see 37 CFR 1.27).
<input type="checkbox"/> Drawing(s)	Number of Sheets		<input type="checkbox"/> Other (specify) _____

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The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☐ No

☒ Yes, the name of the U.S. Government agency and the Government contract number are: DTRA 01-03-C-0047

Respectfully submitted,

SIGNATURE

DATE: 02/26/2004

TYPED OR PRINTED NAME Lynn Astle

REGISTRATION NO. _____ (if appropriate)

☐ Additional inventors are being named on separately numbered sheets attached hereto

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